

APPLICATION

FOR

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on

STAGE AND PLATFORM FOR BUILDING A BIOCHIP, AND BIOCHIP

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# STAGE AND PLATFORM FOR BUILDING A BIOCHIP, AND BIOCHIP

## BACKGROUND OF THE INVENTION

### 1. Field of the Invention

The present invention relates to devices and methods for detecting and analyzing nucleic acids, proteins, macromolecules and other haptens. In particular, the invention includes a biochip. The invention is further directed to devices which the biochip comprises. Methods for making and using biochips and the devices which compose the biochip are also subjects of the present invention.

### 2. Background of the Invention

Nucleic acid probe technology has application in detection of genetic mutations and related mechanisms, cancer screening, determining drug toxicity levels, detection of genetic disorders, detection of infectious disease and genetic fingerprinting. Nucleic acid-based probe methods offer several advantages over conventional microbiological or immunological methods for detection of organisms, as described by Nakamura and Bylund (J. Clinical Laboratory Analysis, 6, 73-83, 1992). Utilizing biochip or microarray technology, one can conduct massively parallel experiments in the areas of genetics and proteomics in applications as diverse as pharmacogenomics, gene expression, compound screening, toxicology, Single Nucleotide Polymorphism (SNPs) analysis, Short Tandem Repeats (STRs) and molecular diagnostics.

Methods to amplify the number of copies of the nucleic acid available for detection of the signal generated after hybridization of the nucleic acid probe have been utilized. A review of nucleic acid based detection methods and various amplification schemes such as polymerase chain reaction (PCR), ligase chain reaction (LCR), transcription based amplification, cycling probe reaction, Q.beta. replicase, and strand displacement amplification may be found in M. J. Wolcott, Clinical Microbiology Reviews, 5, October 1992, pp 370-386.

U.S. Pat. No. 5,175,270 describes an amplification reagent consisting of layers of nucleotide polymers containing double stranded and single stranded sections. Each section has an end which is capable of hybridizing with another molecule.

Probe or hybridization assays are often based on the attachment of an oligonucleotide probe to a surface in order to capture a target nucleic acid molecule (analyte) from a sample. The attachment of this probe to the surface may be through covalent bonds or through a variety of passive absorption mechanisms (e.g., hydrophobic or ionic interactions).

U.S. Pat. No. 5,279,955 describes an immobilization process which uses a heterofunctional cross-linker for a plastic support. The cross-linker consists of a central ring which is hydrophobic and interacts with the plastic, and a hydrophilic chain which terminates in a group capable of reacting with a nucleic acid. Covalent attachment is achieved through a succinyl-olivetol-N-hydroxysuccinimide.

U.S. Pat. No. 5,262,297 describes immobilization of a probe through co-polymers which contain reactive carboxylic acid groups and an 8-50 atom spacer with two or more unsaturated groups within the spacer.

5 U.S. Pat. No. 5,034,428 describes an immobilization process for probes in which a monomer is joined onto a hydrophilic solid support which can be irradiated in an oxygen free atmosphere. This method provides for covalent attachment of the probe.

U.S. Pat. No. 4,806,546 also describes an immobilization process for an amide modified nylon. The method relies on an amidine linkage under anhydrous conditions in the presence of an alkylating agent.

Maskos and Southern, 20 Nucleic Acid Research 1679, 1992, describe a linker system for the attachment of a nucleic acid to a glass support. The linker system allows for the chemical synthesis of a strand of nucleic acids on the surface. The primary advantage of the linker is that it is stable to an ammonia treatment which is required in the synthesis of the polynucleotide. A hexaethylene glycol spacer is incorporated into the linker which attaches to the glass through a glycidoxypropyl silane which terminates in a primary hydroxy group. The silane is condensed onto silane groups on a solid support. Additional cross-linking may be obtained by introducing water so that the epoxide group is cleaved to a diol. An acidic solution facilitates this process. The length of the linker may be varied by changing the spacer to

ethylene glycol, pentaethylene glycol, etc.

Nucleic acid probes that have hybridized to their target sequence are detected based on various methods that introduce a detectable chemiluminescent, fluorescent or other identifiable label into a nucleic acid probe. Several of these techniques are described in U.S. Pat. Nos. 4,968,602, 4,818,680, 5,104,791, and 5,272,056, and International applications WO91/00926 and GB2169403A.

U.S. Pat. No. 5,283,174 (Arnold et al.), describes the use of a chemiluminescent label with DNA probes. The label is composed of an acridinium ester and has a number of desirable properties. It is stable to hybridization conditions, light is emitted only upon exposure to an alkaline peroxide, the emission kinetics are rapid, and the label on the unhybridized probe can be destroyed without an impact on the signal generated by the hybridized probe.

U.S. Pat. No. 5,089,387 describes a diffraction assay for the detection of DNA hybridization. In this invention, a solid support, generally silicon or polysilicon, is coated with a DNA probe. These surfaces are required to inherently adhere the DNA probe to the surface. Once the surface is coated with the probe, the surface is selectively inactivated to provide a series of very strictly controlled reactive probe lines for the generation of the diffraction grating. The unreacted surface is required to be non-light disturbing. The diffraction grating is only generated upon the addition of the analyte to the surface. The angle of diffraction is a

function of the wavelength of incident light and the density and spacing of the individual gratings on the surface. A single detector or a multiple detector array may be used to detect and measure the light from all desired orders of the diffracted light.

5 U.S. Pat. No. 6,060,237 describes a light reflecting assay for the detection of nucleic acid hybridization. In the invention, an optically active solid support capable of producing a thin film effect is coated with an amplifying probe reagent able to bind to the target nucleic acid and create an increase in mass change on the optically active surface without disrupting the thin film effect. The direct optical thin film detection methods of the invention  
10 are extremely sensitive to changes in mass at the surface of the optically active support.

Mixed phase systems have typically been used to perform hybridization assays. In mixed phase assays the hybridizations are performed on a solid phase such as nylon or nitrocellulose membranes. For example, the assays usually involve loading a membrane with a  
15 sample, denaturing the DNA or creating single stranded molecules, fixing the DNA or RNA to the membrane, and saturating the remaining membrane attachment sites with heterologous nucleic acids to prevent the probe reagent from adhering to the membrane in a non-specific manner. All of these steps must be carried out before performing the actual hybridization.

20 Recent development of new technologies for synthesizing or depositing nucleic acids on substrates at very high densities have allowed the miniaturization of nucleic acid arrays yielding increased experimental efficiency and information content. (Lockhart &

Winzler, Genomics, Gene Expression and DNA Arrays, Functional Genomics, 405:6788, Jun 2000, 827-35). Utilizing these Biochips or Microarrays, one can conduct massively parallel experiments in genomics and proteomics with applications as diverse as, pharmacogenomics, gene expression, compound screening, toxicology, Single Nucleotide Polymorphism (SNPs) analysis, Short Tandem Repeats (STRs) and molecular diagnostics. It would be desirable to create a simple, cost-effective device that would produce a low background with optically clean fluorescent detection, allow oligonucleotides to be easily coupled in an array format, and be easily adapted to an automated manufacturing process.

#### SUMMARY OF THE INVENTION

The present invention is, in part, directed to a biochip, a platform which composes the biochip, and a stage which composes the platform. The platform comprises a stage.

The stage of the invention includes a carrier. The carrier has a first side which is hydrophilic and optically inactive. Disposed on the first side of the carrier is an aqueous matrix. The matrix is for attaching or embedding cross-linking agents, at least at the top surface of said matrix. The cross-linking agents attached to the top surface are for cross-linking sensing elements to the top surface of the matrix. The sensing elements are reactive to one or more analytes. Addition or attachment of cross-linking agents to at least the top surface of the stage matrix thereby forms the platform of the invention.

The platform is for building a biochip. The platform includes the stage. In other words, the platform has a carrier having a first side which is hydrophilic and optically inactive. An aqueous matrix is disposed on the first side for embedding cross-linking agents, at least at the top surface of said matrix. The cross-linking agents are for cross-linking sensing elements to the top surface of said matrix, the sensing elements being reactive to one or more analytes. The platform includes cross-linking agents attached to or embedded in the top surface of the matrix, and for cross-linking said sensing elements. Attachment of sensing elements to the cross-linking agent at least to the top surface of the platform thereby forms the biochip of the invention.

The biochip of the invention is for sensing analytes in a test or target sample. The biochip includes the platform of the invention. The platform includes the stage of the invention. Accordingly, the biochip of the invention includes a platform. The platform has a carrier having a first side which is hydrophilic and optically inactive. An aqueous matrix disposed on said first side for embedding cross-linking agents, at least at the top surface of said matrix. The cross-linking agents are for cross-linking sensing elements to the top surface of said matrix, said sensing elements being reactive to one or more analytes in a target or test sample. Included in the platform are cross-linking agents, attached to or embedded in at least the top surface of the matrix. Further included in the biochip are sensing elements cross-linked to the cross-linking agents in the top surface of said matrix.



Other objects, features, and advantages of the present invention will become more fully apparent from the following detailed description of preferred embodiments, the claims, and the accompanying drawings.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a perspective view of a stage comprising a single level matrix;

Figure 2 is a cross-sectional view of a stage at A-A comprising a single level matrix;

Figure 3 is a cross-sectional view of a platform comprising a single level matrix;

Figure 4 is a cross-sectional view of a biochip comprising a single level matrix,  
showing nucleic acid sensing elements cross-linked to the platform of Figure 3;

Figure 5 is a cross-sectional view of a stage at A-A comprising a three level matrix;

Figure 6 is a cross-sectional view of a platform comprising a three level matrix;

Figure 7 is a cross-sectional view of a biochip comprising a three level matrix, showing  
nucleic acid sensing elements cross-linked to the platform of Figure 6.

Figure 8 is a cross-sectional view of a biochip comprising a three level matrix, showing  
fluorescent markers attached to the nucleic acid sensing elements;

Figure 9 is a cross-sectional view of a biochip comprising a three level matrix, showing  
nucleic acid reporter probes hybridized to the nucleic acid sensing elements;

Figure 10 is a cross-sectional view of a biochip comprising a three level matrix,  
showing nucleic acid reporter probes hybridized to the nucleic acid analytes of interest, which  
are hybridized to the nucleic acid sensing elements.

## Reference Numerals in the Drawings

	1	stage comprising a single level matrix
	2	carrier
	3	first side
5	4	hydrophilic bridging agent
	5	matrix
	6	top surface
	7	light blocking agent (\\\\\\\\)
	10	platform comprising a single level matrix
10	15	cross-linking agent
	20	biochip comprising a single level matrix
	25	nucleic acid sensing element
	30	stage comprising a three level matrix
	31	bottom level of matrix
15	32	middle level of matrix
	33	top level of matrix
	40	platform comprising a three level matrix
	50	biochip comprising a three level matrix
	65	marker
20	75	nucleic acid reporter probe
	85	nucleic acid analyte of interest
	A-A	axis upon which cross-sectional views are taken

## MODES OF CARRYING OUT THE INVENTION

### General Description and Definitions

5 The practice of the present invention will employ, unless otherwise indicated, conventional biochemistry, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g. Maniatis et al., *Molecular Cloning: A Laboratory Manual* (1982); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis*, (Gait, N., ed., 1984); *Nucleic Acid Hybridization*, B. Hames & S. Higgins, eds., (1985); Sambrook, J. et al. vol. 1-3, *Molecular Cloning, A Laboratory Manual*, 1989; Harwood, A.J., ed. (1996) *Basic DNA and RNA Protocols*, Humana Press, NJ; Glover, D.M. and Hames, B.D. (1995) *DNA Cloning: A Practical Approach*, 2nd ed. vol. 1-4, IRL Press; Kriegler, M. (1990) *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY; *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, ed. Robert A. Meyers, VCH Publishers, Inc, (1995).

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20 Further reference is made to standard techniques, materials, and equipment in biochips, and electronics for detecting, monitoring, and processing optical or electrical characteristic associated with the binding of analytes to sensing elements disposed on biochips. Factors, techniques, and equipment involved in biochip construction, performance and application of biochips to diagnostics, research, health care, control of industrial processes, environmental monitoring are explained fully in the literature. The electroanalytical methods of optico-analytical transducers and device construction are disclosed and explained in standard

references ("*Microarray Biochip Technology*," Ed. Mark Schena, Eaton Publishing, Natick, MA, 2000; literature available from Hamamatsu Corporation, Bridgewater, NJ). Also available in the literature are methods for optimizing performance factors: selectivity, linear range, calibration, reproducibility, response time, lifetime and the factors affecting biochip performance (e.g. pH, buffers, methods and materials for immobilizing sensing elements. See, e.g., *Biosensors - An Introduction*, (1996), John Wiley & Sons Ltd.; Kress-Rogers, E., ed., *Handbook of Biosensors and Electronic Noses, Medicine, Food and the Environment* (1997), CRC Press; Bickerstaff, G.F. ed., (1997) *Immobilization of Enzymes and Cells*, Humana Press, Inc., Totowa, NJ.; *Bioconjugate Techniques*, Greg T. Hermanson, Academic Press, San Diego, CA, (1996).

The following terminology will be used in accordance with the definitions set out below in describing the present invention.

### Biochip

As used herein, the term "biochip" refers to a device of the present invention comprising sensing elements cross-linked to the top surface of the matrix of a platform (as defined below). The sensing elements possess a selective affinity for analytes of interest within a test sample. As described herein, a biochip of the invention includes a platform, the platform including a stage. For example, an embodiment of the biochip comprises a platform upon which is disposed an array of nucleic acid sensing elements used to examine gene activity or expression, identify gene mutations using a hybridization reaction between the sensing

elements on the surface of the biochip and a target nucleic acid analyte. By way of example but not limitation, depending on the assay and needs of the user, sensing elements may be chosen to distinguish mutant from normal alleles, and/or to distinguishing gene expression products from mutant and normal alleles. Similarly, sensing elements may be chosen to distinguish gene expression in normal tissue from gene expression in diseased tissue.

### Platform

As used herein, the term "platform" refers to a device of the present invention which composes or is a component of the biochip of the invention. The platform of the invention comprises a stage (as defined below) and cross-linking agents embedded at least at the top surface of the matrix of the stage. The cross-linking agents cross-link sensing elements to the top surface of the matrix, thereby forming a biochip.

### Stage

As used herein, the term "stage" refers to a device of the present invention which composes or is a component of the platform of the invention. The stage of the invention comprises a carrier having a first side which has been rendered hydrophilic and optically inactive. Disposed over the first side is a matrix capable of embedding cross-linking agents at least at the top surface of the matrix, thereby forming a platform for building a biochip.

## Sensing Element

The term "sensing element" refers to a macromolecule for detecting an analyte.

The sensing element of the invention includes macromolecules preferentially selective or having a selective binding affinity for an analyte of interest. In assays and other protocols described herein which employ colorimetric and/or radioactive markers and/or reporters, the reaction between sensing element and analyte of interest generates a detectable optical event which results in a signal that can be monitored by an optical reader. Sensing elements include, but are not restricted to:

1. Synthetic or biologically produced nucleic acid (i.e. target specific capture probe or ligand binder probe) which by design contain specific, complimentary nucleotide sequences that allow the capture probe to hybridize or bind to an analyte of interest, for example a target nucleic acid sequence. The capture probe may be deoxyribonucleic acid (DNA), ribonucleic acid (RNA), oligonucleotides, polymerase chain reaction products, or a combination of these nucleotides (chimera), which may be synthesized chemically, isolated from a biological source, or cloned. A nucleic acid sensing element, i.e. target capture probe, may be a linear strand or may contain branch points to increase the density of immobilized probe. The capture probe may provide single or multiple copies of the sequence complimentary to the target nucleic acid. The capture probe is selected to specifically hybridize or bind a target nucleic acid. When more than one capture probe is used in a hybridization assay,

each probe should recognize a unique sequence or ligand separated apart from other target sequence(s) or ligands with the target molecules.

2. Proteins. As used herein, a "target specific capture probe" also refers to synthetic or biologically produced ligand-binding amino acid sequences that allow the protein capture probe to recognize and bind to a target analyte. Protein capture probes, which include enzymes, may be naturally occurring or synthetic, and employed in their unaltered or isolated state or as aggregates with other species. Examples of receptors functioning as capture probes which can be employed by this invention, include, but are not restricted to, antibodies, cell membrane receptors, antibodies (monoclonal or polyclonal), and anti-sera reactive with specific antigenic determinants (such as on viruses, cells, or other materials), drugs, peptides, co-factors, lectins, polysaccharides, cells, cellular membranes, and organelles. Other examples of receptors which can be investigated by this invention include but are not restricted to:

- a. microorganism receptors
- b. enzymes
- c. antibodies
- d. catalytic polypeptides
- e. hormone receptors
- f. opiate receptors

Cross linking systems for attaching sensing elements that are proteinacious to the top surface of the matrix of the biochip are well known in the art (*Bioconjugate Techniques*, Greg T. Hermanson, Academic Press, San Diego, CA, (1996).

5 It should be understood that any macromolecule capable of specifically recognizing and binding to an analyte may be a sensing element of the invention. Such macromolecules (also known in the art as bioaffinity agents) rely on molecular recognition of an analyte as the first step in the generation of a response by a biochip. Strategies for selecting the appropriate bioaffinity agent for an analyte of interest are well known in the art (See Chapter 3 in Kress-Rogers, E. 1997, *Handbook of Biosensors and Electronic Noses: Medicine, Food, and the Environment*, [ed., Kress-Rogers, E.], CRC Press, Boca Raton). A sensing element is further defined to be any macromolecule that when activated by the binding of a chemical ligand or specific analyte, in combination with the addition of appropriate optical labels, causes generation of a detectable optical signal, and operates to communicate the presence of said ligand or analyte from the sample comprising analytes or ligands for detection through an optical reader. The sensing elements of the invention are capable of detecting thousands of natural and synthetic molecular species (analytes). In principal, the range of chemical structures that can be detected by sensing elements in the device and methods of the invention is unlimited (Kress-Rogers, E. 1997, *Handbook of Biosensors and Electronic Noses: Medicine, Food, and the Environment*, [ed., Kress-Rogers, E.], CRC Press, Boca Raton). According to the conditions of the detection method, the binding of the analyte to the sensing element yields a colorimetric event (e.g fluorescence, phosphorescence) that is, a signal, which,



when processed or monitored by an optical reader indicates the presence or concentration of an analyte of interest in the target sample.

The term sensing element is used herein to include a plurality or array of different macromolecules respectively specific for a plurality of specific analytes; or a plurality or array of different macromolecules specific for a single analyte.

#### Scope of Analytes Detectable by Device and Method of the Invention

It should be understood that the scope of analytes in aqueous solutions of interest which can be detected by the sensing elements in the device and methods of the invention is extremely large. Known sensing elements respond to a vast number and variety of analytes, which include but are not limited to those enumerated in Beer, H. In, *Handbook of Biosensors and Electronic Noses: Medicine, Food and the Environment* (1997) CRC Press, pp. 521-532; Alone, E., In, *Hanbook of Biosensors and Electronic Noses: Medicine, Food and the Environment* (1997) CRC Press, pp. 503-519. Nevertheless, they are readily detected and discriminated. The inherent molecular specificity in recognizing and discriminating myriads of foreign compounds should be understood to be a characteristic of sensing element macromolecules, which, using manufacturing techniques well known in the art described above, are design-engineered as sensing elements disposed on the devices of the invention.

### Analyte or Ligand

As used herein, the terms analyte and ligand are used interchangeably. A ligand is a molecule that is recognized by a particular sensing element. The agent bound by or reacting with a sensing element is called a "ligand," a term which is, by definition, meaningful only in terms of its counterpart sensing element. The term "ligand" does not imply any particular molecular size or other structural or compositional feature other than that the substance in question is capable of binding or otherwise interacting with the sensing element. The ligand may serve either as the natural ligand to which the sensing element binds, or as a functional analogue. Examples of ligands that can be investigated by this invention include, but are not restricted to, oligonucleotides, nucleotides, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones, hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs (e.g. opiates, steroids, etc.), lectins, sugars, oligosaccharides, proteins, and antibodies (monoclonal and/or polyclonal).

### Hybridization

As used herein, "hybridization" is the process by which two partially or completely complementary strands of nucleic acid are allowed to come together, under predetermined reaction conditions, in an antiparallel fashion to form a double stranded nucleic acid with specific and stable hydrogen bonds. The nucleic acid capture probe sequence is selected to specifically interact with the target molecule or analyte at a pre-determined degree of stringency. The probe length is pre-determined to provide the required specificity at the degree of stringency used in the assay. Assay conditions are set so that the stringency of

hybridization between the capture probe and the target nucleic acid provides the required specificity.

#### Target/Test Samples

As used herein, "target samples" or "test samples" refer to aqueous aliquots which comprise one or more analytes of interest for detection when brought into contact with the sensing elements. Target samples include without restriction, biological or non-biological samples. Biological samples include, but are not restricted to fluids, cells, subcellular components, and all other biological matter derived from animals, plants, microorganisms, and viruses. Non-biological samples refer to liquid and gaseous environmental samples, which may also include biological matter; and to samples derived from process stream monitoring in the food and non-food industries.

#### Optical Inactivity or Optically Inactive

As used herein, "optically inactive" means a material or surface thereof which produces low reflectivity, low fluorescence, and low scatter properties for optimal optical detection of the target molecules in the present invention. A carrier having a side which is optically inactive, according to this invention, has been otherwise rendered so that optical activity emitted from or through the surface of the carrier is substantially or completely quenched.

The carrier of the invention may be rendered optically inactive such as by disposing one or more light blocking agents directly to the surface of the carrier or mixed with the matrix disposed on the carrier. Alternatively, the carrier may inherently possess optically inactive properties, such that rendering by light blocking agents is not necessary.

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#### Colorimetric Event/Fluorescence

In colorimetric-event detection, and in particular fluorescence-detection methods, a fluorescent molecule has the ability to absorb photons of energy at one wavelength and subsequently emit the energy at another wavelength. Fluorescence is caused by incident radiation impinging upon or exciting an electron of a molecule. The electron absorbs the incident radiation and is raised from a lower quantum energy level to a higher one. The excess energy is released as photons of light as the electrons return to the lower, ground-state energy level. See "*Bioconjugate Techniques*," Greg T. Hermanson, Academic Press, San Diego, CA, (1996), and "*Principles of Biochemistry*," Lehninger, et al., 2<sup>nd</sup> ed., 1993, for a detailed description of fluorescent labeling and detection techniques.

Devices and methods for detecting or reading fluorescence caused by the capture of an analyte(s) of interest by a sensing element mounted on the carrier of the invention are well known in the art ("*Microarray Biochip Technology*," Ed. Mark Schena, Eaton Publishing, Natick, MA, 2000; "*Bioconjugate Techniques*," Greg T. Hermanson, Academic Press, San Diego, CA, (1996); "*Principles of Biochemistry*," Lehninger, et al., 2<sup>nd</sup> ed., 1993, for a detailed description of fluorescent labeling and detection techniques.) References on

fluorescent tags, labels, or markers include Lakowicz, J.R. (ed.) (1991) "Topics in Fluorescence Spectroscopy," vols. 1-3. Plenum, New York; Bright, F.V. (1988) Bioanalytical applications fo fluorescence spectroscopy. *Anal. Chem.* 60, 1031A; Dewey, T.G. (ed.) (1991) "Biophysical and Biochemical Aspects of Fluorescence Spectroscopy." Plenum, New York;

5 McGown, L.B., and Warner, I.M. (1990) Molecular fluorescence, phosphorescence, and chemiluminescence spectroscopy. *Anal. Chem.* 190, 255R; Ploem, J.S., and Tanke, H.J. (1987) "Introduction to Fluorescence Microscopy." Oxford Univ. Press, London;

Darzynkiewicz, Z., and Chrissman, H.A. (eds.) (1990)Flow cytometry. *Methods Cell Biol.*;

Haughland, R.P. (1991) Fluorescent labels. in "Biosensors with Fiberoptics" (D.L. Wise and L.B. Wingard, eds.), pp.85-109. Humana Press, Totowa, New Jersey; and Waggoner, A.S. (1990) Fluorescent probes for cytometry. in "Flow Cytometry and Sorting" (M.R. Melamed, T. Lindmo, and M.L. Mendelsohn, eds.), 2<sup>nd</sup> ed., pp. 209-225. Wiley-Liss, New York.

#### Markers/Reporter Probes

15 As used herein, a "marker" is any agent that can be used to label proteins, nucleic acids, and other molecules as disclosed in the context of the present invention. One of skill in the art selects appropriate markers required to suit the detection method used and needs of the assay. A "reporter probe" refers to a labeled molecule that yields a colormetric event upon exposure to excitation energies. The reporter probes of the present invention possess a

20 selective affinity (i.e., a tendency to react or combine with atoms or compounds of different chemical constitution (See Hawley's Condensed Chemical Dictionary, 13<sup>th</sup> ed., Richard L.

Lewis (rev.), International Thomson Publishing, Inc., NY, 1997)) for the analytes of interest within the test sample.

It should be understood that the terms "marker" and "reporter probe" in context may be used interchangeably. Since each marker has its own colormetric character, more than one labeled molecule, each tagged with a different marker, can be used at the same time to detect two or more analytes of interest. Markers known in the art that are useful for the present invention include, but are not limited to, Cyanine 3 (Cy3), Cyanine 5 (Cy5), and those enumerated in *"Microarray Biochip Technology,"* Mark Schena (ed.), Eaton Publishing, Natick, MA, 2000; *"Bioconjugate Techniques,"* Greg T. Hermanson, Academic Press, San Diego, CA, (1996); *"Principles of Biochemistry,"* Lehninger, et al., 2<sup>nd</sup> ed., 1993; *"Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology,"* Shan-Rong Shi, Jiang Gu, Clive R. Taylor (eds.), Eaton Publishing, Natick, MA, 2000; *"Immunological Reagents & Solutions: A Laboratory Handbook,"* Bassam B. Damaj, Eaton Publishing, Natick, MA, 2000; *"Protein Staining and Identification Techniques,"* Robert C. Allen, and Bruce Budowle, Eaton Publishing, Natick, MA, 1999; *"Affinity and Immunoaffinity Purification Techniques,"* Terry M. Phillips, and Benjamin F. Dickens, Eaton Publishing, Natick, MA, 2000.

#### Light Blocking Agents/Signal-to-Noise Ratio

As used herein, "light blocking agents" refer to agents or treatments that render a surface optically inactive thereby mitigating, preventing, minimizing, reducing background

fluorescence emanating from the carrier, which thereby optimizes the signal to noise ratio for the detection method, e.g. fluorescence detection.

"Signal-to-noise ratio" quantifies how well one can resolve a true signal from the noise of the system. It is typically computed by taking the peak signal divided by the variation in the signal. If a microarray detecting system has a poor signal to noise ratio, the variation in the signal alone can prevent accurate quantitation of each spot (*"Microarray Biochip Technology,"* Mark Schena (ed.), Eaton Publishing, Natick, MA, 2000).

#### Reader/Detector

As used herein, a "reader or detector" refers to any device used to measure the signal generated by the binding of analytes of interest to the sensing elements. Readers or detectors of optical signals, including fluorescence, are well known in the art and adapted for reading biochips (*"Microarray Biochip Technology,"* Mark Schena (ed.), Eaton Publishing, Natick, MA, 2000).

#### Carrier

A variety of materials and formats, depending on the needs of the user, are suitable for forming the carrier. The carrier may be formed of a material that is rigid or flexible, and may be reflective or transmissive. A wide range of rigid materials may be used to form the carrier, including one or more of glass, silicon, fused silica, plastic, ceramic, metal, semiconductor materials, and combinations thereof. The carrier may be of any thickness

desired. Flexible carriers include, but are not restricted to thin sheets of polyester, polystyrene, polyethylene, paper, woven and non-woven textile and like materials.

### Matrix

As used herein, the term "matrix" refers to a medium wherein something is formed, develops, embedded, attached or enclosed. The matrix of the invention is preferably a meshwork of solubilized or suspended agents. The meshwork of solubilizable agents is largely polysaccharide in nature. It should be understood that the matrix coating may be any agent that protects the integrity of the cross-linking agents. The matrix is capable of embedding the cross-linking agents, which cross-links the sensing elements to the surface of the matrix. Materials useful as matrix coatings include, but are not limited to gelatin, agarose, polyacrylamide and blends of the above.

### Cross-Linking Agents

A cross-link is a covalent bond between two macromolecular moieties, usually formed when two macromolecular free radicals combine. Chemical modifications or conjugation processes to achieve cross-links involve the reaction of one functional group with another, resulting in the formation of a covalent bond. The creation of bioconjugate reagents with reactive or selectively reactive functional groups forms the basis for simple and reproducible cross-linking or tagging of target molecules (*"Bioconjugate Techniques,"* Greg T. Hermanson, Academic Press, San Diego, CA, (1996)).



As used herein, "cross-linking agents" include, but are not limited to homobifunctional linkers, heterobifunctional linkers, and zero-length cross-linkers. Homobifunctional linkers are linkers with two reactive sites of the same functionality, such as glutaraldehyde. These reagents could tie one protein to another by covalently reacting with the same common groups on both molecules. Heterobifunctional conjugation reagents contain two different reactive groups that can couple to two different functional targets on proteins and other macromolecules. For example, one part of a cross-linker may contain an amine-reactive group, while another portion may consist of a sulfhydryl-reactive group. The result is the ability to direct the cross-linking reaction to selected parts of target molecules, thus garnering better control over the conjugation process. Zero-length cross-linkers mediate the conjugation of two molecules by forming a bond containing no additional atoms. Thus, one atom of a molecule is covalently attached to an atom of a second molecule with no intervening linker or spacer. One of ordinary skill in the art would refer to "*Bioconjugate Techniques*," Greg T. Hermanson, Academic Press, San Diego, CA, (1996), for a detailed description of cross-linking agents.

In the present invention, cross-linking agents are physically suspended on or in supporting material (matrix) which serves to immobilize or attach or embed the sensing elements held within and at the top surface of the matrix.

A preferred cross-linking agent involves avidin. Without limitation, avidin can be streptavidin, modifications, thereof, or avidin. Avidin can be coupled with agarose by

various chemistries. Ideally, strepavidin is embedded in the surface layer of the matrix coating, binding essentially irreversibly to biotinylated sensing elements (e.g. sensing elements which are nucleic acids). See "*Bioconjugate Techniques*," Greg T. Hermanson, Academic Press, San Diego, CA, (1996). Strepavidin (or any protein containing a lysine) will react with the aldehyde of Nufix™, a commercially available aldehyde-activate matrix. Cross-linking system for linking a proteinaceous capture probe to the platform of the invention are well known, *ibid*.

### **STAGE FOR BUILDING A PLATFORM**

Referring to Figures 1, 2, and 5, in one aspect, the invention involves a stage (1, 30) for building or fabricating a platform (10, 40). The platform, as described in detail below, is useful as a component for forming the biochip (20, 50) of the invention. The stage (1) has a carrier (2) which has a first side (3). In one embodiment, the first side (3) has been rendered hydrophilic by a hydrophilic bridging agent (4), and optically inactive by a light blocking agent (7).

As described in detail below, an embodiment of the stage (1) involves a single-level matrix (5), disposed on the first side (3) of the carrier (2). In preferred embodiments of the stage (30), the matrix (5) involves two or more levels (31, 32, 33) (Fig. 5). In any case, except in which the carrier (2) is inherently optically inactive, any level of the matrix (5) may contain a light blocking agent (7) to control elevation of the background signal. One of skill in

the art will select the appropriate concentration of light blocking agent (7) required to control the signal-to-noise ratio to suit the detection method used and needs of the assay.

5 Addition of a cross-linking system, i.e. cross-linking agents (15), to at least the top surface (6) of the matrix (5) thereby forms a platform (10, 40). The cross-linking agents (15), which are attached or embedded to the top surface (6) of the platform (10, 40), cross-link sensing elements (25) to the top surface (6) of the matrix (5), thereby forming a biochip (20, 50) from the platform (10, 40). One of skill in the art will select the appropriate cross-linking agents (15) and amounts thereof required to cross-link sensing elements (25) to the top surface (6) of the matrix (5) to suit the detection method used and needs of the assay.

10 The carrier (2) may be formed in various *formats* from a variety of materials depending on the needs of the user. The carrier (2) may be formed of a material that is rigid or flexible, and which, depending on the embodiment, may be optically active or optically inactive. A wide range of rigid materials may form the carrier, including glass, silicon, fused silica, plastic, ceramic, metal, and semiconductor materials. The carrier may be of any thickness desired to suit the detection method used and needs of the assay. Flexible carriers include, but are not restricted to thin sheets of polyester, polystyrene, polyethylene, paper, woven and non-woven textile and like materials.

20 Carrier formats may be formed by cutting, sawing, scribing, laser scribing, shearing, or otherwise forming the carrier into the desired size or shape depending on the

reaction conditions, detection method, and detection system used. The carrier can be formed into a broad range of geometric planar configurations circumscribed as polygonal, circular, and free form. A typical geometric shape would be a circle or square (Fig. 1). For example, one suitable geometric configuration of the carrier for a single use assay is a rectangle, 0.50 inch x 1.0 inch. Another example is a square, 0.50 inch by 0.50 inch. Carrier sizes are not restricted to the above, as alternative formats and assays may require substantially more or less reactive test surface.

The material selected for the carrier (2) may either be inherently hydrophilic or may require rendering such that the surface of the first side (3) is rendered hydrophilic. Hydrophilicity is defined as having a strong tendency to bind or absorb water, which results in swelling and formation of reversible gels (*Hawley's Condensed Chemical Dictionary*, 13<sup>th</sup> ed., Richard L. Lewis (rev.), International Thomson Publishing, Inc., NY, 1997). It is desirable to render the first side (3) or the surface of the first side of the carrier (2) hydrophilic to allow adhesion of an aqueous matrix (5) coating to the carrier (2). Various methods are known in the art to render surfaces hydrophilic. In an embodiment of the invention, a hydrophilic bridging agent (4) is applied to the surface of the first side (3) of carrier (2). A typical hydrophilic bridging agent is gelatin. Alternatively, the carrier may undergo Corona Ion treatment, in which the surface is ionized with a charge that is stronger than the hydrophobic properties of the carrier. Various carrier materials may be purchased pre-coated with gelatin from the Dupont Corporation. One of ordinary skill in the art would refer to Markgraf, D.A., "Corona Treatment: An Overview," 1986 Coextrusion Conference Proceedings, TAPPI PRESS,

Atlanta, p. 85; Marra, J.V., "Surface Modification of Polypropylene Film." 1985 Polymers, Laminations and Coatings Conference Proceedings, TAPPI PRESS, Atlanta, p. 103; Thompson, K., "Surface Treatments for Coex Polymer Films/Coatings," 1989 Coextrusion Conference Proceedings, TAPPI PRESS, Atlanta, p. 11; literature available from Enercon Industries Corporation, Germantown Wisconsin, for principles, methods, and materials for preparing hydrophilic surfaces.

An aqueous matrix (5) is disposed on the hydrophilic first side (3) of the carrier (2). A matrix is a medium wherein something may be formed, developed, or embedded. The matrix (5) of the invention is preferably a meshwork of solubilized or suspended agents. The meshwork of solubilizable agents is largely polysaccharide in nature. Materials useful as matrix coatings include, but are not limited to gelatin, agarose, polyacrylamide and blends of the above. A preferred matrix material is agarose. One of skill in the art will select the appropriate matrix material required to suit the detection method used and needs of the assay. Depending on the material selected for the matrix, it may be desirable to add one or more reagents to achieve chemical stability and/or optimal pH for the desired assay.

Preferably, surfactants are added to the matrix (5) to enhance the aqueous properties of the matrix to optimize coating of the carrier (2). A surfactant is any compound that reduces interfacial tension between a liquid and a solid (*Hawley's Condensed Chemical Dictionary*, 13<sup>th</sup> ed., Richard L. Lewis (rev.), International Thomson Publishing, Inc., NY, 1997). Surfactants useful in the invention may be ionic, non-ionic, zwitterioninc, or

amphoteric. Preferably, the surfactants include, but are not limited to commercially available Tween, Brij, Triton, sulphonates and the salts thereof, quaternary ammonium compounds, thoxylates, amides, and fatty acids.

5 Preferably, buffers are added to stabilize the pH of the matrix coating for optimal hybridization conditions. See "*Principles of Biochemistry*," second edition, Lehninger, Nelson, and Cox, for details regarding hybridization reaction conditions. Commercially available buffers include, but are not limited to methane-ethane sulphonic acid, phosphate, Tris, and Hepes.

10 In an embodiment, the matrix (5) comprises one or more humectants to reduce or control the shrinking and swelling of the matrix. A humectant is a substance having an affinity for water with stabilizing action on the water content of a material (*Hawley's Condensed Chemical Dictionary*, 13<sup>th</sup> ed., Richard L. Lewis (rev.), International Thomson Publishing, Inc., NY, 1997). It may be desirable to control surface irregularities (e.g. elevations and/or depressions) of the matrix for accurate detection optical signals by a reader. Otherwise, a technician would have to constantly recalibrate the reader to compensate for minute irregularities in the matrix caused by shrinking and swelling of the matrix from the addition of capture probes, i.e. sensing elements (25), aqueous test samples containing target molecules (85), and reporters (75). Albeit a planar surface without irregularities may not be achieved, nonetheless, humectants are useful for minimizing surface irregularities. Suitable humectants include, but are not limited to glycerin, oils, sugars, and some detergents.

In those embodiments of the platform (10, 40) in which the carrier (2) inherently provides stray, background, or otherwise undesirable optical signals, the carrier may be rendered optically inactive such as by application of one or more light blocking agents (7) directly to the first side (3) of the carrier (2) or mixed with the matrix (5) disposed on the carrier. Alternatively, if the material selected for the carrier inherently possesses optically inactive properties, rendering by light blocking agents is optional.

For optically-based detection methods, it is desirable to optimize signal-to-noise ratio. For example, in fluorescence-based assays, it is desirable to add one or more light blocking agents (7) to the matrix (5) coating. In those embodiments of the invention where it is desirable to use polyester as the carrier, optically pure polyester is preferred as impurities found in polyester may fluoresce and cause undesirable background signal or noise. The addition of a light blocking agent (7) to the matrix (5) serves to prevent or minimize these inherent fluorescent qualities from disrupting the detection or reading of the optical signal generated from or as a consequence of the binding of analyte(s) of interest (85) to one or more sensing elements (25). Agents useful as light blocking agents include, but are not limited to iron oxide, titanium dioxide, carbon black, and mixtures of the above. In a preferred embodiment of the stage (30), the matrix includes a sufficient amount of iron oxide to render the carrier (2) optically inactive.

In a more preferred embodiment of the stage (3), the carrier (2) is formed from *hydrophobic*, optically pure Mylar, 100 microns in thickness. Mylar emits a significant amount

of background, stray, or otherwise undesirable optical signal, e.g. fluorescence. Such material is commercially available from the Dupont Corporation and is used widely in the photographic film coating industry. The carrier is provided with a gelatin coating on the first side (3), which acts as a hydrophilic bridging agent (4) to allow attachment of the aqueous matrix (5) coating.

5 In a version of this embodiment, the matrix has three levels (31, 32, 33 in Figs. 5, 6, 7). The bottom level (31) of the matrix (5) is disposed on the gelatin (4), and is a standard agarose in water solution ranging from about 1% to about 5%, with 1% being preferable. The bottom level (31) contains the surfactant Tween-20 and is buffered by HEPES. The bottom level (31) is applied at 200 ml of liquid matrix to 1m<sup>2</sup> of carrier (2) material, which ultimately yields a  
10 wet thickness of 200 microns (dry thickness is approximately 10% of the wet thickness). The middle level (32) is a standard agarose in water solution ranging from about 1% to about 5%, with 1% being preferable. The middle level (32) contains a 3% dispersion of the light blocking agent (7) iron oxide, the surfactant Tween-20 and is buffered with methane ethane sulphonic acid (MES). Like the bottom level (31), the middle level (32) is applied at 200 ml of solution  
15 to 1m<sup>2</sup> of carrier (2), which yields a wet thickness of 200 microns. The middle level (32) serves to block the incident light from penetrating the matrix (5) and contacting the carrier (2), and prevents any optically active impurities within the carrier from causing undesirable background signal or noise, thereby rendering the carrier optically inactive. The top level (33) is an aldehyde-activated agarose in water solution ranging from about 1% to about 5%, with  
20 1% being preferable.



## **PLATFORM FOR BUILDING A BIOCHIP**

Referring to Figures 3 and 6, another aspect of the invention is directed to a platform (10, 40) for building a biochip (20, 50). The platform comprises the stage (1, 30 of Figs. 2 and 5) and cross-linking agents (15) attached to or embedded at least at the top surface (6) of the matrix (5) of the stage. The cross-linking agents (15) cross-link sensing elements (25) to the top surface (6) of the matrix (5), thereby forming a biochip.

The platform (10, 40) is formed by adding a sufficient amount of appropriate cross-linking agents (15) at least to the top surface (6) of the stage. The cross-linking agents (15) allow one or more sensing elements (25), as described below, to attach or cross-link to the top surface (6) of the matrix (5). The sensing elements (25) possess a selective affinity for one or more analytes of interest (85).

As described above, cross-linking agents (15) useful in the invention include, but are not limited to homobifunctional linkers, heterobifunctional linkers, and zero-length cross-linkers. Cross-linking agents (15) are physically suspended on or in the matrix (5) which serve to immobilize the sensing elements (25) held within and at the top surface (6) of the matrix. A preferred cross-linking system is the heterobifunctional group consisting of biotin-streptavidin, i.e. biotinylated sensing elements bound to avidin-coupled agarose. Ideally, streptavidin is embedded within and on the surface of the matrix, binding essentially irreversibly to the biotinylated sensing elements. Such cross-linking agents are commercially available from the Pierce Company and the Aldrich Company.

In a preferred embodiment of the platform (10, 40), the top level (33) of the matrix (5) coated during stage fabrication further includes streptavidin (a bifunctional cross-linker), attached to the top surface (6) of the matrix, dibasic sodium phosphate buffer and a surfactant. The top level (33) involves an aldehyde-activated agarose, commercially available as Nufix™, which reacts with the primary epsilon amine of the lysine group of strepavidin to form a Schiff's Base. This unstable bond is then reduced, with the addition of sodium cyanoborohydride, to yield a stable covalent bond with strepavidin. The unreacted aldehyde in the top level (33) of the matrix (5) is then quenched with histidine.

## **BIOCHIP**

Referring to Figures 4 and 7, another aspect of the invention is directed to a biochip (20, 50). The biochip comprises the platform (10, 40) and sensing elements (25) cross-linked to the cross-linking agents (15) attached to or embedded in the top surface (6) of the matrix (5) of the platform.

### **Sensing Elements**

Sensing elements (25) useful in the invention include macromolecules preferentially selective for an analyte of interest (85). Sensing elements (25) include, but are not restricted to synthetic or biologically produced nucleic acid and synthetic or biologically produced ligand-binding amino acid sequences. It should be understood that the sensing elements (25) selected should possess at least one functional group adapted to covalently bond

with an available functional group of the cross-linker attached to the top surface (6) of the matrix (5), thereby achieving adhesion of the sensing element (25) to the top surface of the matrix. Methods for forming sensing elements possessing at least one functional group, as described in this paragraph, are disclosed herein.

5

In a preferred embodiment of the invention, the sensing elements (25) are biotinylated (i.e., bound to biotin) and are deposited or brought into contact with cross-linking agents (25) (e.g. strepavidin) attached or embedded to the top surface (6) of the matrix (5) of the platform (10, 40). Strepavidin and biotin are an example of a cross-linking system. The biotin binds essentially irreversibly to the strepavidin at the top surface (6) of the matrix (5). Biotinylated sensing elements are commercially available from many sources (e.g. Aldrich, Pierce, Sigma).

#### Depositing Sensing Elements onto the Top Surface of the Platform

Commonly used techniques in the art are available for depositing sensing elements (25) onto the top surface (6) of the matrix (5) of the platform (10, 40). The sensing elements (25) are typically deposited in droplets with a spot diameter ranging from about 20 to 1000  $\mu\text{m}$ , preferably 120  $\mu\text{m}$  spaced about 200  $\mu\text{m}$  apart. The platform (10, 40) is suitable for spotting by all contact and non-contact methods. Spotting devices and techniques known in the art include, but are not limited to, syringe-solenoid, solid pin replicator, quill and split pin, tweezer, Pin-and-Ring™, and jetting and piezoelectric pumps. One of ordinary skill in the art

would refer to "*Microarray Biochip Technology*," Ed. Mark Schena, Eaton Publishing, Natick, MA, 2000, for a detailed description of spotting techniques.

### Reporter Probes

Reporter probes (75) useful in the invention include labeled macromolecules or other analytes which possess a selective affinity, i.e., are preferentially selective, for a sensing element, or, in another embodiment, for an analyte of interest (85), which has bound to the sensing element (25) on the top surface (6) of the matrix (5). Reporter probes (75) include, but are not restricted to synthetic or biologically produced nucleic acid sequences and synthetic or biologically produced ligand-binding amino acids sequences. The reporter probe (75) selected should possess a selective affinity for the analytes of interest (85), bound to the sensing element (25) on the top surface (6) of the matrix (5), thereby achieving adhesion of the marker probe to the analyte of interest.

In a preferred embodiment of the invention, the reporter probes (75) contain either the Cy3 or Cy5 marker (65) and are deposited onto the top surface (6) of the matrix (5) following adhesion of the analyte of interest (85) to the sensing element (25). The reporter probe (75) will react, combine, or otherwise bind to an analyte (85) of interest, thereby causing a colormetric effect upon exposure to excitation energy. This colormetric effect indicates the presence of the analyte of interest (85). Reporter probes that are design-engineered specifically to the user's requirements are commercially available from NEN Life Science Products, Boston, MA.

## EXAMPLES

The following examples are offered by way of illustration, and not by way of limitation.

### EXAMPLE 1

#### 5 Experiment 1 - Marker in Matrix Coating

10 This experiment demonstrated that an aqueous matrix adheres to the carrier, the first side of which having been rendered hydrophilic, and does not interfere with the colormetric event of a fluorescent marker. Optically pure, 100 micron thick Mylar (Dupont Corp., Cat. No, P4C1A) with a gelatin coating was obtained from the Dupont Corporation. A solution of standard agarose in water was prepared as described below. Cy3 marker (NEN Life Sciences) was added to the solution and mixed thoroughly yielding a uniform suspension. The Cy3-agarose solution was then spread evenly over the carrier using a Leneta Wire-Cator (BYK-Gardner Corporation) as described below. The coating was then allowed to cool. Using a Bio-Rad MRC-1024 Confocal Microscope and Omnicrome 643 100 Kr-Ar laser (Bio-Rad Laboratories, Hercules, California), a 200 micron area of the coated carrier was excited with a wavelength of 550 nm. The microscope detected an image over every 200 micron area of the surface of the matrix using a detection (emission) setting of 570 nm.

#### 15 Experiment 2 - Concentration of Light Blocking Agent

20 This experiment determined the amount of light blocking agent required to render the carrier optically inactive. A 2% solution of standard agarose in water and 6 gm of

iron oxide was prepared as described below. The Cy3-agarose coated carrier from experiment 1 was then coated to make a 200 micron layer of the iron oxide-agarose solution and allowed to cool. Using the same procedure from Experiment 1, an image was detected over every 200 micron area of the surface of the matrix. The iron oxide-agarose coating step was repeated five times on the same carrier until no image was detected on the surface of the matrix using the confocal microscope of experiment 1. The total concentration of iron oxide which completely blocked the Cy3 image identified the amount of light blocking agent needed to render the carrier optically inactive. It should be understood that one of skill in the art can determine the amount of any light blocking agent required to render a carrier optically inactive using the above procedure.

### Experiment 3 - Formation of a Stage for Building a Platform

This experiment was performed to show that the total amount of light blocking agent required to render the carrier optically inactive could be applied in a single coating, thereby forming the stage for building a platform. The procedure of experiment 2 was duplicated using a single layer of iron oxide-agarose solution, at the concentration identified in experiment 2. Again, using the confocal microscope and laser of experiment 1, the Cy3 image was completely blocked. The experiment was then repeated utilizing a different piece of Mylar film to verify the results.

#### Experiment 4 - Sensing Element Bound to the Matrix

As used hereinafter,

Oligo A - SEQ ID: 1, biotinylated at 5' position; Cy3 labeled at 3' position.

Oligo B - SEQ ID: 1, biotinylated at 5' position; not labeled at 3' position.

Oligo C - SEQ ID: 2, Cy3 labeled at 3' position.

Oligo D - SEQ ID: 2, biotinylated at 5' position; not labeled at 3' position.

Oligo E - SEQ ID: 2, not labeled at 3' position.

Oligo F - SEQ ID: 3, Cy3 labeled at 3' position.

SEQ ID: 1 = 5'-AAT CCA GAT ATA GTC ATC TAG CAA TAC A-3'

SEQ ID: 2 = 5'-TTA GCT CGA CTC AGG GAT CCG GAT TGT ATT GCT AGA-3'

SEQ ID: 3 = 5'-ATC CGG ATC CCT-3'

This experiment was performed to show that sensing elements adhere to the top surface of the matrix coating containing cross-linking agents. A 2% solution of standard agarose in water with 2.25% strepavidin was prepared as described below. The strepavidin-agarose solution was then coated onto the first side of a clean (i.e., as delivered from Dupont Corporation) piece of carrier. An aliquot of Oligo A (NEN Life Science Products) was then deposited over the surface of the matrix and allowed to cross-link. Using the confocal microscope and laser of experiment 1, an image was detected over every 200 micron area of the surface of the matrix.

#### Experiment 5 - Adhesion of Sensing Element to the Matrix

This experiment was performed to show that the heterobifunctional cross-linking bond between the sensing elements and the surface of the matrix is not affected by

subsequent washes with deionized water. The procedure of experiment 4 was repeated.

However, the coated carrier was repeatedly washed with deionized water following the addition of Oligo A to verify that the sensing element was bound essentially irreversibly to the surface of the matrix. Using the confocal microscope and laser of experiment 1, an image was detected over every 200 micron area of the surface of the matrix.

#### Experiment 6 - Light Blocking Agent

This experiment was performed to show that the light blocking agent disposed over the matrix containing a cross-linking system renders the carrier optically inactive. The solution of agarose in water with strepavidin from experiment 4 was coated onto the first side of a clean piece of carrier. Then the solution of light blocking agent in agarose from experiment 3 was coated over the initial matrix coating. Using the confocal microscope and laser of experiment 1, no image was detected.

#### Experiment 7 - Formation of a Platform for Building a Biochip

This experiment was performed to show that a matrix coating, containing cross-linking agents, disposed over the light blocking agent has no affect on the light blocking agent's ability to render the carrier optically inactive. A standard solution of aldehyde-activated agarose, commercially available as Nufix™, was prepared with strepavidin, as described below. The procedure of experiment 6 was repeated. The Nufix™ solution was then coated over the light blocking agent and allowed to cool, thereby forming a platform for



building a biochip. Using the confocal microscope and laser of experiment 1, no image was detected.

#### Experiment 8 - Labeled Sensing Element Cross-Linked to a Platform

5                    This experiment was performed to show that a fluorescently-labeled sensing element produces a detectable colormetric effect (i.e, fluorescence) when bound to the top surface of a platform. The procedure of experiment 7 was repeated. Then an aliquot of Oligo A from experiment 4 was deposited over the surface of the matrix and allowed to cross-link. Using the confocal microscope and laser of experiment 1, an image was detected over every 200 micron area of the surface of the matrix.

#### Experiment 9 - Unlabeled Sensing Element Produced No Image

15                   This experiment was performed to show that an unlabeled sensing element bound to the surface of the matrix does not produce a colormetric effect. The procedure of experiment 7 was followed using Oligo B (NEN Life Science Products). Using the confocal microscope and laser of experiment 1, no image was detected.

#### Experiment 10 - Detecting a Reporter Probe Bound to a Sensing Element

20                   This experiment was performed to show that a fluorescently-labeled analyte (e.g., nucleic acid) that hybridizes to a sensing element bound to the surface of the matrix produces a colormetric effect. The procedure of experiment 9 was repeated. An aliquot of Oligo C (NEN Life Science Products), which is partially complementary to Oligo A, was then

deposited over the surface of the matrix and allowed to hybridize with Oligo B. This experiment was performed in triplicate, allowing Oligo C to hybridize for 4 hours, 8 hours, and overnight. Using the confocal microscope and laser of experiment 1, images were detected over every 200 micron area of the surfaces of each of the matrices.

## Experiment 11 - Formation of a Biochip

This experiment was performed to show that unlabeled nucleic acid sensing elements spotted onto the surface of the matrix do not produce a colormetric effect. A 210 micron pin was obtained from TeleChem International, Inc., Sunnyvale, California. A corresponding 4-spot metal spotting block was machined at a local metal fabricating shop such that the weight of the pin spotter determines the amount of oligo that is deposited. The procedure of experiment 7 was repeated. Two spots of Oligo B from experiment 9 were then spotted onto the surface of the matrix. Using the confocal microscope and laser of experiment 1, no image was detected. The device was then washed with deionized water and placed in the confocal microscope for another reading. No image was detected.

## Experiment 12 - Detecting Reporter Probes Bound to Spotted Sensing Elements on a Biochip

This experiment was performed to show that the spotted nucleic acid sensing elements bound to the surface of the matrix remain fixed in position and do not migrate over the surface of the matrix. Further, this experiment demonstrated that stray light impinging upon the device from surfaces other than the top did not affect the light blocking agent's ability to render the carrier optically inactive. The procedure of experiment 11 was repeated. An

aliquot of Oligo C (NEN Life Science Products) was then deposited over the surface of the matrix and allowed to hybridize with Oligo B, which was spotted onto the surface of the matrix. After washing with deionized water, two distinct spot images were detected on the surface of the matrix using the confocal microscope and laser of experiment 1. Finally, stray light was allowed to contact the bottom surface of the carrier during another reading with the confocal microscope. The stray light did not affect upon the images detected.

#### Experiment 13 - Nucleic Acid Sandwich Hybridization Assay Performed on the Biochip

A standard nucleic acid sandwich hybridization assay was carried out on the biochip of the invention. The procedure of experiment 11 was repeated. An aliquot of Oligo E (NEN Life Science Products) was then deposited over the surface of the matrix and allowed to hybridize with Oligo B, which was spotted onto the surface of the matrix. After washing with deionized water, an aliquot of Oligo F was deposited over the surface of the matrix and allowed to hybridize with Oligo E. After washing with deionized water, two distinct spot images were detected on the surface of the matrix using the confocal microscope and laser of experiment 1. One of ordinary skill in the art would refer to Sambrook, J. et al. vol. 1-3, *Molecular Cloning, A Laboratory Manual*, 1989 for a detailed description of nucleic acid sandwich hybridization assays.

## **EXAMPLE 2 - FABRICATING THE STAGE**

### **PREPARATION OF REAGENTS**

#### **2% Agarose - Bottom Level of Matrix**

1. Measure 20 grams of dry agarose and place into 2 liter beaker
2. Add 1 liter of deionized water and place on hot plate to slowly bring to a boil
3. Stir occasionally with a glass stir rod until all of the agarose is completely melted
4. Turn off heat and allow mixture to cool to room temperature with aluminum foil cover over beaker to keep out dust.
5. After hardening, the agarose should be labeled and can be tightly sealed and stored in a refrigerator for later use.

#### **2% Agarose with Iron Oxide (Light Blocking Agent) - Middle Level of Matrix**

1. Measure 20 grams of dry agarose and place into 2 liter beaker
2. Add 500 ml of deionized water and place on a hot plate to slowly bring to a boil
3. Stir occasionally with a glass rod until all of the agarose is completely melted
4. Measure 6 grams of iron oxide into container and mix until completely and homogeneously dispersed
5. In a second 2 liter beaker, warm about 750 ml of deionized water to about 80°C
6. Bring the volume of the iron oxide preparation up to 1 liter by adding 80°C deionized water. Mix the dispersion until smooth and completely dispersed
7. Turn off heat and allow mixture to cool to room temperature with an aluminum foil cover over the beaker to keep out dust.

8. After hardening, the agarose should be labeled and can be tightly sealed and stored in a refrigerator for later use.

#### Buffer A

1. Add 100 ml of deionized water to a 250 ml Erlenmyer flask with a small stir bar
2. Place on a magnetic stirrer and stir slowly
3. Add 0.01 moles of Hepes free acid and allow to dissolve
4. Measure pH and adjust to  $7.4 \pm 0.05$  using either 0.5 molar NaOH or 0.5 molar HCl
5. Add 0.02 grams of Tween-20
6. Bring volume in the flask up to 200 ml using deionized water and stir to dissolve contents completely

#### Buffer B

1. Add 100 ml of deionized water to a 250 ml Erlenmyer flask with a small stir bar
2. Place on a magnetic stirrer and stir slowly
3. Add 0.01 moles of methane-ethane sulphonic acid (MES) and allow to dissolve completely
4. Measure pH and adjust to  $6.8 \pm 0.05$  using either 0.5 molar NaOH or 0.5 molar HCl
5. Add 0.03 grams of Tween-20
6. Bring volume in the flask up to 200 ml using deionized water and stir to dissolve contents completely.

## DEPOSITING OR COATING MATRIX ONTO CARRIER

By way of example, but not limitation, a method for depositing the matrix coating onto the first side of the carrier involves quickly dispensing a melted material, suitable for use as a matrix coating, onto the carrier. Just after the solution spreads over the surface of the carrier, a Leneta Wire-Cator or a Bird Film applicator capable of delivering a prescribed volume per square meter is drawn down the carrier to spread liquified matrix material evenly over the surface of the carrier. Such coating applicators are commercially available from BYK-Gardner Corporation. The carrier sat at room temperature until the coating was dry. Various other useful coating techniques are known to those skilled in the art.

By way of further example, but not limitation, another method for depositing or coating the first side of the carrier involves industrial machinery. Flexible carrier materials are commercially available from sources such as Dupont Corporation. A flexible material is rolled onto a core suitable for loading onto said industrial machinery. The machinery draws the carrier material under a series of warmers, coaters and driers, depending on the product desired. Standard film coating techniques are known to those of skill in the art.

### Bottom Level of Matrix

1. Heat about 1.5 liters of water in a beaker until approximately 60°C and pour into a 1 liter graduated cylinder. Place a wire wound coating rod capable of delivering a 50 ml per square meter coverage into the cylinder in order to warm it.

2. Cut a piece of 5.25 inch gelatin-coated mylar (polyester) carrier about 20 inches long and tape one short edge onto a flat surface such as a lab bench. Be certain that the hydrophilic side (gelatin coated side) is facing up. This can be determined by simply breathing on each side. The hydrophilic side will fog up with breath.

5

3. Use a spatula and measure 10 grams of 2% agarose into a conical centrifuge/culture tube
4. Add 10 ml of Buffer A.
5. Place the loosely capped tube into a microwave and heat on high in 20 second increments until melted. Be careful not to boil over the tube.
6. Place the tube in the water bath at 50°C and hold until needed
7. Working quickly, remove the wire wound rod from the warm water and dry with a paper towel. Place at the top of the carrier.
8. Remove the culture tube from the water bath and pour about ½ of the contents over the coating rod. Just after solution spreads onto the carrier, lightly grab the rod and draw down the coating.

20

9. Allow the coated carrier to sit at room temperature for 20 minutes. It can then be allowed to air dry for at least 2 hours.

#### Middle Level of Matrix

- 5
1. Heat about 1.5 liters of water in a beaker until approximately 60°C and pour into a 1 liter graduated cylinder. Place a wire wound coating rod capable of delivering a 200 ml per square meter coverage into the cylinder in order to warm it.
  2. Tape down the carrier (upon which the bottom matrix level has been deposited or coated), coating side up, onto a flat surface such as a lab bench.
  3. Use a spatula and measure 10 grams of 2% agarose with Iron Oxide into a conical centrifuge/culture tube.
  4. Add 10 ml of Buffer B
  5. Place the loosely capped tube into a microwave and heat on high in 20 second increments until melted. Be careful not to boil over the tube.
  6. Place the tube in the water bath at 50°C and hold until needed
- 20



7. Working quickly, remove the wire wound rod from the warm water and dry with a paper towel. Place at the top of the coated carrier.
8. Remove the culture tube from the water bath and pour about ½ of the contents over the coating rod. Just after solution spreads onto the carrier, lightly grab the rod and draw down the coating.
9. Allow the coated carrier to sit at room temperature for 20 minutes. It can then be allowed to air dry for at least 2 hours.

### **EXAMPLE 3 - FABRICATING THE PLATFORM**

#### **PREPARATION OF REAGENTS**

##### **2% Nufix™ - Top Level of Matrix**

1. Measure 20 grams of Nufix™ and place into a 2 liter beaker
2. Add 1 liter of deionized water and place on a hot plate to slowly bring to a boil
3. Stir occasionally with a glass rod until all of the agarose is completely melted
4. Turn off heat and allow mixture to cool to room temperature with an aluminum foil cover over the beaker to keep out dust.
5. After hardening, the agarose should be labeled and can be tightly sealed and stored in a refrigerator for later use.

### Buffer C

1. Add 100 ml of deionized water to a 250 ml Erlenmyer flask with a small stir bar
2. Place on a magnetic stirrer and stir slowly
3. Add 1.0mM of dibasic sodium phosphate
- 5 4. Measure pH and adjust to  $5.8 \pm 0.05$  using either 0.5 molar NaOH or 0.5 molar HCl
5. Bring volume in the flask up to 200 ml using deionized water and stir to dissolve contents completely

### Buffer D

1. Add 100 ml of deionized water to a 250 ml Erlenmyer flask with a small stir bar
2. Place on a magnetic stirrer and stir slowly
3. Add 2.0mM of dibasic sodium phosphate
4. Measure pH and adjust to  $7.0 \pm 0.05$  using either 0.5 molar NaOH or 0.5 molar HCl
5. Bring volume in the flask up to 200 ml using deionized water and stir to dissolve contents completely.

## DEPOSITING CROSS-LINKING SYSTEM ONTO STAGE TO FORM PLATFORM

### Top Level of Matrix

1. Use a spatula and measure 10 grams of 2% Nufix™ into a conical centrifuge/culture tube.
2. Add 2 ml of Buffer C.

3. Place the loosely capped tube into a microwave and heat on high in 20 second increments until melted. Be careful not to boil over the tube.
4. Place the tube in the water bath at 50°C and hold until needed.
5. In a 10 ml disposable culture tube, prepare 3.0 ml of a 10 mg/ml solution of Streptavidin in Buffer C.
6. Warm the Streptavidin solution to 50°C in a water bath.
7. After the Streptavidin solution has warmed, remove 2.5 ml and add it to the tube from step 4 above. Mix the 14.5 ml Nufix™ / Streptavidin solution and incubate for 2 hours in a 50°C water bath.
8. While the Nufix™ / Streptavidin solution reacts, prepare the following:
  - 8a. In a 10 ml disposable conical tube, add 1 ml of 1.33M sodium cyanoborohydride to 1ml Buffer D solution;
  - 8b. In a second 10 ml disposable conical tube, add 1.5 ml of 300mM histidine to 1.5 ml Buffer D solution;

9. After the 2 hour incubation of the Nufix™ / Streptavidin solution, add 1.67 ml of 300mM histidine. Mix thoroughly and incubate at 50°C for 1 hour.
10. After 1 hour of incubation, add 0.29 ml of 1.33M sodium cyanoborohydride. Add 0.04 gm Tween-20 and mix thoroughly and react for 1 hour at 50°C. Do not cap the tube tightly as hydrogen gas may be produced. Allow tube to vent.
11. Heat about 1.5 liters of water in a beaker until approximately 60°C and pour into a 1 liter graduated cylinder. Place a wire wound coating rod capable of delivering a 200 ml per square meter coverage into the cylinder in order to warm it.
12. Tape down the stage, coating side up, onto a flat surface such as a lab bench.
13. Working quickly, remove the wire wound rod from the warm water and dry with a paper towel. Place at the top of the coated carrier.
14. Remove the culture tube from the water bath and pour about ½ of the contents over the coating rod. Just after solution spreads to onto the carrier, lightly grab the rod and draw down the coating.
15. Allow the coated stage to sit at room temperature for 20 minutes. It can then be allowed to air dry for at least 2 hours.

#### **EXAMPLE 4 - PREPARATION OF THE BIOCHIP**

Recent development of new technologies for synthesizing or depositing nucleic acids on substrates at very high densities have allowed the miniaturization of nucleic acid arrays yielding increased experimental efficiency and information content. (Lockhart & Winzeler, Genomics, Gene Expression and DNA Arrays, Functional Genomics, 405:6788, Jun 2000, 827-35). The methods disclosed herein are easily adaptable to an automated manufacturing process ("*Microarray Biochip Technology*," Ed. Mark Schena, Eaton Publishing, Natick, MA, 2000); and reference to standard film coating procedures available from the film or film coating industry.

Commonly used techniques in the art are available for depositing sensing elements onto the surface of the platform. The sensing elements are typically deposited in droplets with a spot diameter ranging from about 20 to 1000  $\mu\text{m}$ , preferably 120  $\mu\text{m}$  spaced about 200  $\mu\text{m}$  apart. The platform is suitable for spotting by all contact and non-contact methods. Spotting devices and techniques known in the art include, but are not limited to, syringe-solenoid, solid pin replicator, quill and split pin, tweezer, Pin-and-Ring™, and jetting and piezoelectric pumps. One of ordinary skill in the art would refer to "*Microarray Biochip Technology*," Ed. Mark Schena, Eaton Publishing, Natick, MA, 2000, for a detailed description of spotting techniques.

### **EXAMPLE 5 - USE OF THE BIOCHIP**

Using the biochips or microarrays of the invention, one can conduct applications in the fields of genomics and proteomics such as pharmacogenomics, gene expression, mutation analysis, compound screening, toxicology, Single Nucleotide Polymorphism (SNPs) analysis, Short Tandem Repeats (STRs) and molecular diagnostics. The biochip of the invention is easily adaptable to massively parallel experimentation in these applications. One of skill in the art will employ conventional biochemistry, molecular biology and recombinant DNA techniques, antigen retrieval techniques, as well as techniques and protocols in molecular diagnostics referred to herein, and, to the extent possible, incorporated by reference herein. Such techniques are explained fully in the literature.

The embodiments of the invention are not meant to be restricted by the above disclosure, but only by the claims below. Nonetheless, the present invention finds use as a platform for a variety of different detection methods. The detection modes include, but are not limited to reflection, fluorescence, phosphorescence, absorption, magnetism, polarization, chemiluminescence, electricity, conductivity, resistivity, colormetricity, piezo-electricity, and radioactivity.

Utilizing the biochips of the invention as microarrays, one can conduct massively parallel experiments in applications as diverse as, pharmacogenomics, gene expression, compound screening, toxicology, Single Nucleotide Polymorphism (SNPs) analysis, Short Tandem Repeats (STRs) and molecular diagnostics. The biochip finds use in

massively parallel experimentation in these applications. Embodiments of the biochip of the invention include arrays of peptides, proteins, small molecules, mRNAs, cDNAs, clones, tissues, and cells disposed on the biochip as sensing elements. In short, the devices and methods of the invention are advantageously used in exploring genome data, protein function in relation to genome function (proteomics), genetic determinants (Nature Insight, vol. 405, no. 6788, 15 June 2000).

Further embodiments involve adapting the invention for use in a cartridge, said cartridge being used in an automated detection system based on fluorescence-detection methods.